THE EFFECTS OF 2,4,5-TRICHLOROPHENOXYACETIC ACID AND 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN ON DEVELOPING CHICKEN EMBRYOS

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SUMMARY

Analytical grade 2,4,5-T and TCDD are dissolved in acetone and injected into the airspace of fertile chicken eggs. Toxicity studies suggest that the LD_{50} for 2,4,5-T is 133.1 $\pm$ 3.4 mg of 2,4,5-T per kg of egg weight. The LD_{50} for TCDD was estimated to be $2.4 \times 10^{-4} \pm 1.97 \times 10^{-4}$ mg of TCDD per kg of egg weight. A partitioned chi-square analysis of the embryo viability data and the nonparallel dose response lines obtained from 2,4,5-T and TCDD suggest an independent mode of action for the two chemicals.

As a result of the injection of these two chemicals, an increase in the liver weight to egg weight percentages occurred. The possibility of enlargement due to elevated DNA and RNA levels was ruled out as both DNA and RNA levels were depressed per gram of liver tissue. RNA to DNA ratios remained the same as controls so any alternation of nucleic acid content was rejected as the cause of liver weight increase.

CHAPTER I

INTRODUCTION

2,4,5-trichlorophenoxyacetic acid (2,4,5-T) is a herbicide that is widely used in the control of brush and broadleafed weeds. A chlorinated hydrocarbon first registered on March 2, 1948 by the Amchem Company of Ambler, Pennsylvania (MacLeod, 1970), the use of this compound has been frequent and varied. As a result of this, a great deal of information concerning the use of 2,4,5-T has been amassed.

Most extensively used in the mid-sixties, 2,4,5-T is still a useful chemical even though it has come to be regarded as potentially dangerous by the general public. Rights-of-ways and waterways may be kept clear of vegetation by aerial application of 2,4,5-T dissolved in diesel fuel or some other petroleum distillate. Noxious weeds such as poison ivy and poison oak are also effectively controlled by similar applications. The primary agricultural use of this herbicide is the treatment of rangeland and pastureland to kill undesirable vegetation that competes with grass for water and nutrients. Treatment of grazing land with this method is preferable to more expensive manual removal of brush and weeds by such methods as hoeing, cutting, or burning.

Civilian use of 2,4,5-T dropped in the mid-sixties, but production continued on a large scale to meet military demands.

2,4,5-T mixed with equal parts of another closely related herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was used extensively during the Vietnam conflict. This compound, referred to as Agent Orange, was employed to defoliate areas with suspected enemy activity.

With such widespread usage, 2,4,5-T came to be included in a group of pesticidal chemicals that underwent a screening by the National Cancer Institute in 1964. Results of this investigation showed 2,4,5-T to be both teratogenic and toxic to laboratory rats and mice (in MacLeod, 1970). In light of this report and the continued increasing usage of 2,4,5-T, Courtney in 1969 conducted studies to determine the teratogenic and toxicologic capabilities of this chemical. This study showed an increased incidence of cystic kidney and cleft palate in mice and abnormalities such as increased liver weight to body weight ratios and gastrointestinal tract hemmorrhage in rats when administered orally or subcutaneously (Courtney, 1970).

As a result of the Courtney study and pressure from several governmental agencies in 1969, the United States Food and Drug Administration made an effort to alleviate the problems associated with 2,4,5-T. Since 2,4,5-T had proven to be an economically important chemical, complete restriction of usage did not occur. Agricultural use was limited to non-crop species and military application was restricted to nonpopulated areas. Further research was also initiated in order to

establish guidelines regulating the use and manufacture of this herbicide.

Subsequent studies by Courtney and Moore (1971) and Sparchu et al. (1971) were conducted after it was discovered that 2,4,5-T (Figure 1) used in earlier work contained 30 milligrams (mg) of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) per kilogram (kg) of 2,4,5-T. TCDD (Figure 2) is a by-product formed in the synthesis of 2,4,5-T and is one of the most teratogenic and toxic chemicals known to man. The profound effects noted in Courtney's prior study were attributed to this impurity.

The chemical TCDD has been known since 1957 and the effects of it characterized somewhat; however, it was not until 2,4,5-T production peaked in the mid-sixties that levels of TCDD were found to be unacceptably high. By 1966, sufficient technology was available to keep levels of TCDD below one part per million during the synthesis of 2,4,5-T. The first pieces of information concerning this subject came when it was reported that a chicken crop was destroyed as a result of having ingested TCDD contaminated fat (Allen and Carstens, 1966). In this report, an accumulation of serious fluid in the pericardial sac of these chickens was characterized and the causative agent was labelled "chick edema factor." Other researchers including Higgenbotham et al. (1968) and Grieg et al. (1972) have suggested that this "factor" is TCDD. Greig also reported finding jaundiced livers in treated

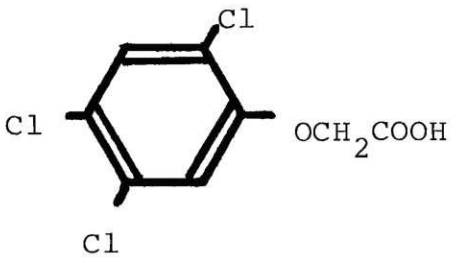

Figure 1. 2,4,5-T

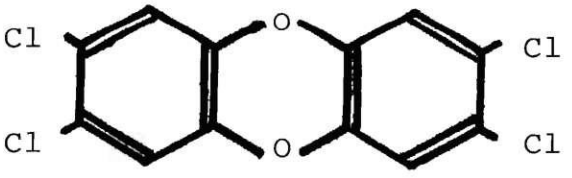

Figure 2. TCDD

chicken embryos with accompanying increased liver weight to body weight ratios. Increased liver weights in mice administered TCDD orally were reported by Goldstein et al. (1973) and Vos et al. (1973).

The reason for liver enlargement is unclear and information concerning this enlargement is incomplete. Greig et al. (1973) has suggested an increase in lipid content of the liver is responsible. Increased cell numbers (hyperplasia) as discussed by Goldberg (1966) might also cause liver enlargement. Another possible explanation of liver enlargement is increased cell size (hypertrophy). Several studies indicate that 2,4,5-T and TCDD alter enzyme systems, causing increased RNA synthesis in some. Greatly increased RNA synthesis could cause an increase in cell mass and work initiated by the finding of Vos that TCDD is porphyrogenic in mice supports this. Hepatic porphyria may be induced by several drugs; all are found to stimulate the activity of the initial enzyme in heme synthesis, δ-amino-levulenic acid synthetase (ALA synthetase) (Granick, 1966). This enzyme stimulation is thought to represent increased induction, which could result in greater liver weight. Poland and Glover (1973) have reported a dose response relationship between TCDD and ALA synthetase activity in developing chicken embryos. Other enzyme systems have also been found to be altered upon administration of TCDD to experimental animals. Greig (1972) has presented evidence that TCDD stimulates the enzymes that detoxify zoxazolamine in the rat.

In the present study, the developing chicken embryo will be examined to determine if any increase in liver weight is found as a result of 2,4,5-T or TCDD administration. The livers will also be examined to determine if an increase in cell size (hypertrophy) or cell numbers (hyperplasia) occurs.

2,4,5-T and TCDD toxicity studies on the chicken embryo are incomplete. Strange and Kerr (1976) have studied the effect of 2,4,5-T on developing chicken embryos, but neither TCDD nor the possibility of interaction between the two chemicals was considered. Courtney's 1971 study briefly considers the possibility of synergism, but the effect was not demonstrated when mice were administered doses of 2,4,5-T and TCDD simultaneously. LD_{50} values for the chicken embryo will be calculated for both 2,4,5-T and TCDD and the possibility of interaction between the two chemicals examined.

CHAPTER II

MATERIALS AND METHODS

The chicken embryo was selected as the experimental animal for several reasons: (a) the egg is a closed system which reduces the risk of laboratory contamination, (b) fertilized chicken eggs are cheap and readily available, (c) large numbers of eggs may be easily dosed, and (c) maintenance of incubating eggs is minimal, not requiring feeding or changing of litter. Chicken eggs used in this study were obtained from KimberCHIK Hatcheries of Dixie (Atlanta, Georgia) and were of the Shaver-Starcross White-Leghorn strain. Weights of the eggs ranged from 43.1 grams to 70.8 grams.

Analytical standards of 2,4,5-T (lot number AGR86187) and TCDD (lot number 851:144-II) were supplied by the Dow Chemical Corporation (Midland, Michigan) and were both in the form of white crystalline solids.

Since the method of administration affects the toxicity of a compound (Mawdesley-Thomas, 1973), several possibilities were considered. The Sauter and Steele (1971) method of feeding hens low levels of pesticides and then measuring egg hatchability was rejected on the grounds it was too complex and indirect. Another method in which the dose was administered to the embryo was sought. The method of Khera and Lyon (1967) in which the pesticide is injected directly into the yolk sac

was also rejected. This procedure was undesirable because the yolk of an eighteen day old embryo is not completely incorporated into the body and the high viscosity of the yolk would tend to slow diffusion of the herbicide. The method used by Gebhardt (1972) in which the herbicide is deposited into the airspace of the egg was finally selected.

The eggs in this study were first weighed and a dental burr fitted in a Dremel hobby drill (model 260) was used to drill a small hole in the blunt end of the egg allowing access to the airspace. The dose was computed (Appendix A) and administered with a Hamilton fifty microliter syringe (Reno, Nevada) after which the hole was sealed with paraffin (Paraplast).

Gebhardt maintains this is the most sensitive method of determining the LD_{50} of eggs while at the same time causing the least amount of trauma to the developing embryo.

The airspace of an egg is small; therefore a vehicle which would carry a sufficient experimental dose in a small volume was required. Since 2,4,5-T and TCDD are not readily soluble in many substances (water, corn oil, and propylene glycol), this problem was not easily solved. Strange and Kerr found that dimethyl sulfoxide (DMSO) was not an entirely satisfactory vehicle, because at higher experimental doses, it was felt that DMSO contributed somewhat to reduced hatchability. It was found that acetone would dissolve enough 2,4,5-T and TCDD to carry the required dose in a volume that could easily

be placed in the airspace. Being a neutral organic solvent that is found in the body as a by-product of β-oxidation, acetone was considered best suited for this study.

A 0.75 molar solution of 2,4,5-T in acetone gave volumes of 5.8 to 55.6 microliters (ul) for the lightest egg with lowest dose to heaviest egg with greatest dose. The average volume of a 2,4,5-T dose was approximately 36 ul.

A 6.3×10^{-9} molar solution of TCDD in acetone was used as a stock solution from which serial dilutions were made. Volumes of these dilutions ranged from 15.3 to 20.3 ul depending upon egg weight and dose. The average volume of a TCDD dose was approximately 17 ul.

Eggs were incubated in the "Favorite Incubator," a forced-draught wooden incubator manufactured by Leahy Manufacturing Company, Incorporated (Higginsville, Missouri). Constant temperature was maintained with a YSI "Thermistemp" temperature controller (model 70A, Yellow Springs Instruments Company, Yellow Springs, Ohio). Standard incubation conditions as recommended by the manufacturer were maintained throughout the incubation period (Appendix C).

Karnofsky (1955) has determined that most developmental defects induced in embryos are compatible with embryo viability through day eighteen of incubation. Since this study considers both toxicological and teratogenic effects, eighteen days of incubation were considered a sufficient period of development. The embryo at this time can be examined visually to determine

if it is viable and the liver is large enough to be easily removed. For these reasons, percent embryo viability at day eighteen of incubation was used as the index of toxicity instead of percent hatch.

Toxicity Evaluation

To determine the percent embryo viability of the eggs being used in this study, untreated eggs were incubated on four occasions to give a grand total of 78 control eggs. To determine if the injection of acetone or the drill vibration reduced viability, 23 eggs were drilled and injected with the volume of acetone that would be required to carry the maximum dose of 2,4,5-T used. All eggs in the study were dosed on day zero of incubation.

2,4,5-T doses were administered as milligrams of herbicide per kilogram of egg weight. Originally, three groups of 24 eggs each received doses of 25, 50, and 75 mg/kg of 2,4,5-T. Subsequently, four more groups of 18 eggs each received 50, 75, 100, and 150 mg/kg.

TCDD doses were administrered as milligrams of impurity per kilogram of egg weight. Doses of 6.65×10^{-4}, 6.65×10^{-5}, 6.65×10^{-6}, and 6.65×10^{-7} mg/kg were administered to four groups of 24 eggs each. These doses represent amounts of TCDD that would be found in an LD_{50} of 2,4,5-T if TCDD were present at 5, 0.5, 0.05, and 0.005 mg per kg of 2,4,5-T. The TCDD contamination levels used in this study are in the range of levels

actually found in commercial preparations of 2,4,5-T.

Teratogenic Evaluation

After each egg was opened on day eighteen of incubation to examine for viability of the embryo, the liver was dissected out. The gall bladder was removed and the livers were placed in metal weighing pans and weighed. Weights were recorded with treatment numbers and the pans were immediately covered with aluminum foil and placed in an ice and acetone bath to keep the tissues cold. As soon as possible, the samples were transferred to a freezer (-10°C) and stored for future quantification of DNA and RNA.

Total DNA and RNA was extracted from ten livers in each treatment group by the method of Mejbaum (1939). Quantification of DNA and RNA was done spectrophotometrically using a Beckmann model 25 digital spectrophotometer at 260 nanometers (nm). Calf thymus DNA and yeast RNA were used as standards.

Statistical Methods

LD_{50} values were determined using linear regression equations. Two equations were generated using the results of the toxicity studies of 2,4,5-T and TCDD. Correlation coefficients were calculated to determine the significance of the two lines.

Results of embryo viability studies were analyzed by chi-square tests using 2 x 2 contingency tables (Sokal and Rohlf, 1969).

A slight modification of chi-square analysis was used to evaluate the possibility of interaction between 2,4,5-T and TCDD. The exact partitioning of the chi-square statistic in an R x C contingency table has been treated by Kimball (1954). The statistics obtained by such partitioning may be used to test orthogonal contrasts of a particular type, each with one degree of freedom. The resulting component chi-square values computed will add exactly to the total chi-square computed in the usual way (Kastenbaum, 1960). The data obtained from this study fit a 2 x 3 contingency table (Appendix C).

Analysis of the effect of treatment on DNA, RNA, RNA/DNA rations, and liver weight to egg weight ratios was done by Student-Newman-Keuls multiple range test.

CHAPTER III

RESULTS AND DISCUSSION

Results of tests conducted to determine the toxicity of acetone are shown in Table 1. Separate groups of control eggs were incubated on four different occasions to give 73 of 78 viable embryos or 93.59 percent viability. The amount of acetone that was injected into the acetone treatment eggs represented 80 percent of the total volume of a 150 mg/kg dose of 2,4,5-T (see Appendix A). A 150 mg/kg dose of 2,4,5-T was the largest dosage of the herbicide administered during the course of the study and more acetone was required to carry this level of herbicide than any other dose administered. A 91.3 percent viability was obtained from the acetone injected group.

A 70 percent hatch was reported in toxicity studies conducted by McLaughlin et al. (1963) when 50 ul of acetone were injected into the airspace of fertilized chicken eggs. The 91.30 percent embryo viability of this study seems somewhat inconsistent with this information; however, several differences in methodology should be pointed out. The volume of acetone administered in the present study was based on egg weight and did not exceed 44.5 ul. Egg weight was not considered in the doses administered in the study by McLaughlin. The index of toxicity in this study was percent embryo

Table 1. Effect of Drilling and Injecting Volume of Acetone Required to Carry 150 mg/kg Dose on Embryo Viability

Treatment	Number of Eggs	Number of Viable Embryos	Percent Viable
Control	12	12	100.00
	24	20	83.33
	24	23	95.83
	18	18	100.00
Total	78	73 } *	93.59
Acetone Injection	23	21 }	91.30

*X^2 n.s., ldf at $p < 0.05$ level

viability, not percent hatch as in the work by McLaughlin. The possibility of an embryo being viable at day 18 of incubation yet not being able to free itself from the egg at day 22 cannot be overlooked.

With chi-square analysis of the difference between the control and acetone injected groups not significant at the $p < 0.05$ level, it was felt that acetone was a suitable solvent for use in this study. These results also suggest that neither drill vibration nor the injection procedure had an adverse effect on the developing embryo.

The linear regression line for 2,4,5-T is shown in Figure 3. The data points used to fit the regression line and to calculate the regression equation with are shown in Table 2. The LD_{50} for 2,4,5-T using the equation $Y = 111.176 - 0.4597X$ was calculated to be 133.1 $\pm$ 3.4 milligrams of 2,4,5-T per kilogram of egg weight. A chi-square analysis of the calculated viability and the viability of those eggs dosed at 133 mg/kg was not significantly different at the $p < 0.05$ level (Table 4). Strange and Kerr estimated an LD_{50} of 62 mg 2,4,5-T per kg of egg weight using DMSO as a vehicle. The suggestion that DMSO is an unsuitable vehicle is supported by the large discrepancy between these two calculated 2,4,5-T LD_{50} values.

The same methods were used to calculate the data for TODD. The data points used to calculate the regression equation are shown in Table 3. Figure 4 shows these points graphed

Table 2. Data Points Used to Calculate the LD_{50} for 2,4,5-T Using Linear Regression

Dose (mg/kg)	Number Viable/ Number Dosed	Percent Viability
25	23/24	95.83
50	39/42	92.86
75	35/42	83.33
100	10/18	55.56
150	8/18	44.44

Regression Equation: $Y = 111.176 - 0.4597X$ ($r = 0.9573$)

$LD_{50} = 133.1 \pm 3.4$ mg/kg (n=5)

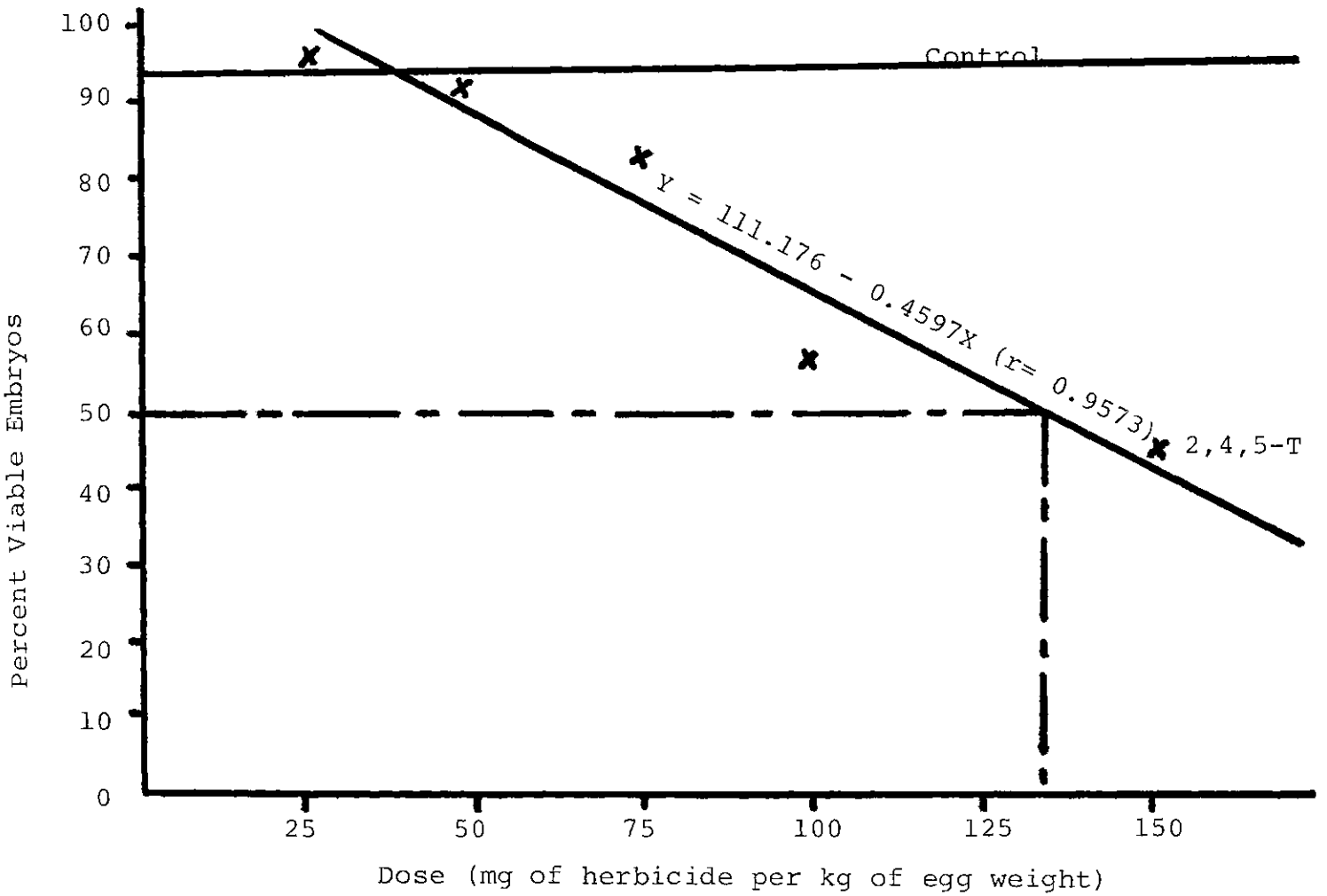

Figure 3. Percent Viable Embryos vs. Dose of 2,4,5-T.

Table 3. Data Points Used to Calculate the LD_{50} for TCDD Using Linear Regression

Dose (mg/kg)	Number Viable/ Number Dosed	Percent Viability
6.65×10^{-4}	5/24	20.83
6.65×10^{-5}	14/24	58.33
6.65×10^{-6}	16/24	66.67
6.65×10^{-7}	17/24	70.83

Regression Equation: $Y = 67.1326 - 7.029 \times 10^{4}X$ ($r = 0.9808$)

$LD_{50} = 2.4 \times 10^{-4} \pm 1.97 \times 10^{-4}$ mg/kg ($n = 4$)

Table 4. Viability of Treatment Groups

Treatment	Number Viable/ Number Dosed	Percent Viability
Control	73/78	93.59
2,4,5-T (133 mg/kg)	16/36[b]	44.44
TCDD (6.65×10^{-6} mg/kg)	18/24 } [c]	75.00
2,4,5-T ÷ TCDD[a]	3/24 } [c]	12.50

[a] LD_{50} dose of 2,4,5-T plus 6.65×10^{-6} mg/kg TCDD

[b] $X^2 = 0.2222$, n.s., ldf at $p < 0.05$ level (tested against calculated LD_{50} viability)

[c] $X^2 = 13.5684$, ldf at $p < 0.005$ level

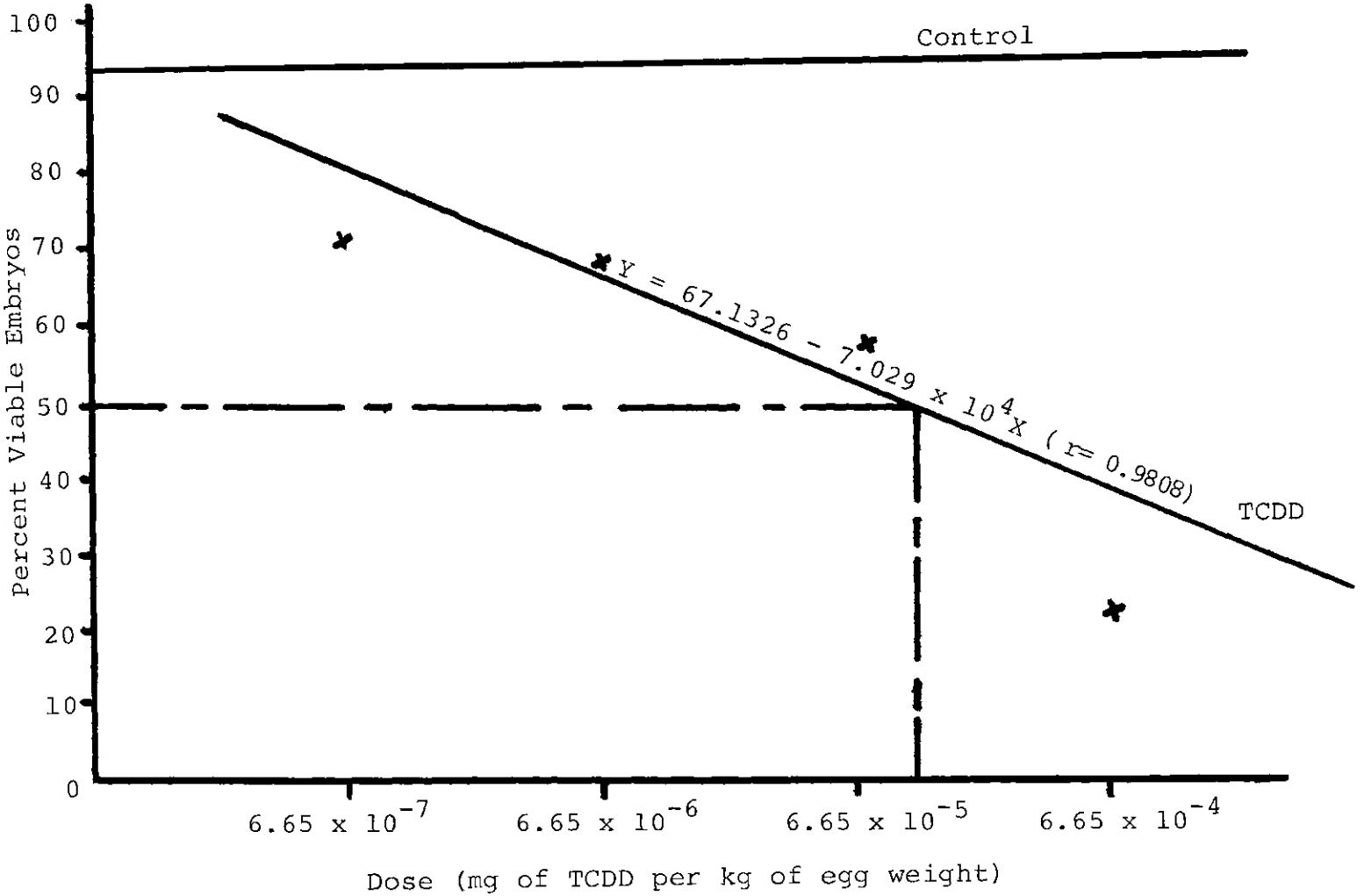

Figure 4. Percent Viable Embryos vs. Dose of TCDD.

along with the regression line. The equation that resulted from these data points is $Y = 67.1326 - 7.029 \times 10^{4}X$ and the LD_{50} calculated from this equation was $2.4 \times 10^{-4} \pm 1.97 \times 10^{-4}$ milligrams of TCDD per kilogram of egg weight. This amount of TCDD represents a contamination level in 2,4,5-T of approximately 1.8 parts per million (ppm). The actual quantity of TCDD that would be injected into a 52 gram egg is 0.013 micrograms. Although dosing was not done by egg weight, Higgenbotham et al. have reported an LD_{100} of 0.05 microgram per egg which is consistent with the results of this study.

The dose of TCDD administered in the treatment shown in Table 4 was 6.65×10^{-6} mg/kg and resulted in 18 viable embryos from 24 dosed eggs. This represents a 75.00 percent viability which is close to the 66.67 percent viability found in the earlier TCDD LD_{50} calculations (Table 3). A chi-square analysis of the viability data for the two 6.65×10^{-6} mg/kg doses of TCDD in Tables 3 and 4 shows no significance at the $p < 0.05$ level.

Data pertaining to the examination of the possibility of interaction between 2,4,5-T and TCDD are presented in Table 4. The hypothesis that the actions of 2,4,5-T and TCDD on embryo viability are independent and additive is not supported. If the slopes of the dose response curves are not identical there is no reason to suspect that the modes of action of the two chemicals are similar (Loomis, 1974). Statistically, this

premise is supported when the independent viabilities of 2,4,5-T and TCDD (33.33 percent) from Table 4 are compared with the viability (17.50 percent) of the LD_{50} 2,4,5-T and 6.65×10^{-6} mg doses of TCDD. Likewise, if the results of the TCDD injection from the LD_{50} calculation data (Table 3) are evaluated, the same relationship is demonstrated. Results of these chi-square analyses are shown in Appendix C. Therefore, the data support the premise of independent modes of actions for 2,4,5-T and TCDD.

In Table 5 are listed for each treatment group, the means of the following: a) total DNA expressed as micrograms (ug) of DNA per gram (g) of liver weight, b) total RNA expressed as ug of RNA per g of liver weight, c) RNA to DNA ratio, and d) liver weight expressed as a percentage of egg weight.

One of the most noticeable trends in Table 5 is the increase in liver weight to egg weight ratios. Largest increases occur in the treatment groups containing 2,4,5-T. Table 6 shows the Student-Newman-Keuls multiple range analysis of the liver weight to egg weight percentage means. The 2,4,5-T and 2,4,5-T and TCDD mixture groups differ significantly from the control groups. There is an increase in the TCDD and acetone treatment group percentages; however, neither of them are significantly different from control percentage means. The liver enlargement reported by earlier workers (Greig et al., Goldstein et al., and Vos et al.) as a result

Table 5. Means and Standard Errors of Treatment Groups (n=10)

Treatment	DNA (ug DNA/mg of liver wt.)	RNA (ug RNA/mg of liver wt.)	RNA/DNA	Liver wt./Egg wt. (Percent)
Control	2.86 ± 0.20	145.17 ± 6.01	50.76 ± 3.56	0.68 ± 0.03
Acetone[2]	3.15 ± 0.12	152.34 ± 3.72	48.36 ± 1.28	0.72 ± 0.03
2,4,5-T[b]	2.14 ± 0.14	122.85 ± 4.63	57.41 ± 3.87	0.86 ± 0.05
TCDD[c]	2.80 ± 0.10	122.30 ± 3.97	43.68 ± 2.57	0.74 ± 0.03
2,4,5-T[d] + TCDD	2.45 ± 0.10	116.80 ± 3.85	47.67 ± 1.76	0.87 ± 0.04

[a]Volume of acetone required to deliver a 150 mg/kg dose of 2,4,5-T.

[b]LD_{50} dose of 2,4,5-T.

[c]Amount of TCDD present in LD_{50} dose of 2,4,5-T if present at 0.05 parts TCDD per million parts 2,4,5-T.

[d]LD_{50} dose of 2,4,5-T mixed with 0.05 ppm TCDD.

of TCDD administration was not demonstrated in this study. It should be noted that an increase was seen though not statistically significant. Increase in liver weight to egg weight percentages due to acetone may be simply a result of toxic insult to the liver. The profound increase from 2,4,5-T injection cannot be attributed to acetone, because a smaller volume of acetone was used.

Tables 7 and 8 both show trends toward a reduction in liver nucleic acids. The paradoxial increase in nucleic acids as a result of acetone injection suggests a different mode of liver enlargement than that caused by 2,4,5-T. The effect of TCDD on decreasing liver DNA seems to be more subtle than that of 2,4,5-T, yet it depresses RNA levels markedly.

If it is assumed that liver cell DNA remains constant, then from Table 7 it follows that administration of 2,4,5-T and TCDD tends to reduce the number of cells that are required to make one gram of liver. This does not support the contention that hyperplasia or increased cell numbers is responsible for the increase in liver mass. Moreover, the depression of RNA per gram of liver weight (Table 8) tends to refute the hypothesis of Poland and Glover that liver enlargement comes from increased induction of protein synthesis.

In Table 9, comparisons of the RNA/DNA ratios fail to demonstrate a significant difference from the control mean, but the 2,4,5-T ratio was different from all other treatment groups. Referring to Tables 6, 7, and 8, the effect (mean)

Table 6. Comparison of Means by Student-Newman-Keuls Multiple Range Test of Liver Weight to Egg Weight Ratios

Treatment Groups				
Control	Acetone	TCDD	2,4,5-T	Mixture
Means (Liver Weight to Egg Weight)[a]				
0.0068	0.0072	0.0074	0.0086	0.0087

[a]Means not underscores by the same line are significantly different at the $p < 0.05$ level.

Table 7. Comparison of Means by Student-Newman-Keuls Multiple Range Test of ug of DNA per mg of Liver

Treatment Groups				
2,4,5-T	Mixture	TCDD	Control	Acetone
Means (ug of DNA per g of Liver)[a]				
2.14	2.45	2.80	2.86	3.15

[a]Means not underscored by the same line are significantly different at the $p < 0.05$ level.

Table 8. Comparison of Means by Student-Newman-Keuls Multiple Range Test of ug of RNA per mg of Liver

Treatment Groups				
Mixture	TCDD	2,4,5-T	Control	Acetone
Means (ug RNA per g of Liver)[a]				
116.80	122.30	122.85	145.17	152.34

Table 9. Comparison of Means by Student-Newman-Keuls Multiple Range Test of RNA to DNA Ratios

Treatment Groups				
TCDD	Mixture	Acetone	Control	2,4,5-T
Means (RNA to DNA Ratios)[a]				
43.68	47.67	48.36	50.76	57.41

[a]Means not underscored by the same line are significantly different at the $p < 0.05$ level.

of 2,4,5-T tends to be closest to the effect (mean) of the 2,4,5-T and TCDD mixture. In considering all the information available from these statistics concerning liver weight increase, it seems more reasonable to suspect the 2,4,5-T mean in Table 9 of being a poor measure of the true RNA to DNA ratio than to argue that the mean of the mixture group (Table 9) is a result of adding the effects of TCDD and 2,4,5-T.

As a result of these analyses, the liver enlargement caused by 2,4,5-T and TCDD does not appear to be a result of elevated nucleic acid levels. The evidence in this study discounts the possibility of hyperplasia as discussed by Goldberg and points to enlargement of liver size. The possibility of enlargement of cell size as a result of increased RNA levels has also been eliminated. A more plausible explanation seems to be the suggestion of Greig et al. that hypertrophy from increased lipid content causes the liver cell enlargement.

CHAPTER IV

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

As a result of this investigation, the following conclusions are made:

The use of acetone as a solvent did not significantly affect embryo viability at day 18 of incubation, or cause an increase in liver cell DNA or RNA content.

The LD_{50} value for a 0.75 molar solution of 2,4,5-T in acetone was calculated to be 133.1 ± 3.4 mg per kg of egg weight. The LD_{50} value for TCDD was determined by administering serial dilutions of a 6.3×10^{-9} molar TCDD in acetone and was found to be $2.4 \times 10^{-4} \pm 1.97 \times 10^{-4}$ mg per kg of egg weight.

The embryo viability data revealed nonparallel slopes for the 2,4,5-T and TCDD curves. The chi-square evaluation likewise supported the nonadditivity of the two chemicals, and suggested independent modes of action.

The increase in liver weight noted in this study was not the result of elevated liver cell nucleic acid content.

Recommendations

Judicious use of 2,4,5-T while maintaining TCDD contamination levels within acceptable limits should reduce the chance

of future accidents and not deny the public the benefits of a useful chemical. Within safe limits, 2,4,5-T has been found to have no effect on some mammalian species (Dougherty and Herbst, 1975). Further research is called for, however to determine what constitutes "safe" levels.

The question of liver enlargement should also be further investigated. If liver weight increase is not a result of elevated nucleic acid content, other possibilities should be considered. At this point, an investigation into possible lipid level changes in affected livers should be pursued.

APPENDICES

APPENDIX A

INJECTION VOLUME

Injection Volume (ul) By Dose (mg/kg)

Egg Weight (grams)	Dose .75m 2,4,5-T (mg/kg)						TCDD*
	25	50	75	100	133	150	
	(ul)	(ul)	(ul)	(ul)	(ul)	(ul)	(ul)
40	5.1	10.3	15.5	20.6	27.4	30.9	13.3
41	5.3	10.6	15.9	21.1	28.1	31.7	13.6
42	5.4	10.8	16.2	21.7	28.9	32.6	14.0
43	5.5	11.1	16.6	22.2	29.1	33.5	14.3
44	5.7	11.3	17.0	22.8	30.5	34.4	14.6
45	5.8	11.7	17.6	23.4	31.2	35.2	15.0
46	6.0	12.0	18.0	24.0	31.9	36.0	15.3
47	6.1	12.3	18.4	24.6	32.6	36.8	15.6
48	6.3	12.5	18.8	25.0	33.3	37.6	16.0
49	6.4	12.8	19.2	25.6	34.0	38.4	16.3
50	6.5	13.0	19.6	26.0	34.7	39.2	16.6
51	6.7	13.3	20.0	26.6	35.4	40.0	17.0
52	6.8	13.6	20.4	27.2	36.1	40.8	17.3
53	6.9	13.8	20.8	27.6	36.8	41.6	17.6
54	7.0	14.1	21.2	28.2	37.5	42.4	18.0
55	7.2	14.4	21.6	28.8	38.2	43.2	18.3
56	7.3	14.6	22.0	29.2	38.9	44.0	18.6
57	7.4	14.9	22.4	29.8	39.6	44.8	19.0
58	7.6	15.1	22.8	30.2	40.3	45.6	19.3
59	7.7	15.4	23.2	30.8	41.0	46.4	19.6
60	7.8	15.7	23.6	31.4	41.6	47.2	20.0
61	8.0	16.0	24.0	32.0	42.3	48.0	20.3
62	8.1	16.2	24.4	32.4	43.0	48.8	20.6
63	8.2	16.4	24.8	32.8	43.7	49.6	20.9
64	8.4	16.7	25.2	33.4	44.4	50.4	21.3
65	8.5	17.0	25.6	34.0	45.1	51.2	21.6
66	8.6	17.2	25.8	34.4	45.8	51.6	21.9
67	8.7	17.5	26.2	35.0	46.5	52.4	22.3
68	8.9	17.8	26.8	35.6	47.2	53.6	22.6
69	9.0	18.0	27.0	36.0	47.9	54.0	22.9
70	9.1	18.3	27.4	36.6	48.6	54.8	23.3
71	9.3	18.5	27.8	37.0	49.3	55.6	23.6
72	9.4	18.8	28.1	37.6	50.0	56.4	24.4

*6.3×10^{-9} M TCDD Solution for 5ppm 2,4,5-T was diluted by factors of 10 to give .5, .05, and .005 doses, hence volumes remained the same.

APPENDIX B

STANDARD CONDITIONS OF CHICKEN EGG INCUBATION*

1. Incubation period: 21 days
2. Operating temperature: 100°F
3. Wet-bulb reading during turning period: 85-87°F
4. Wet-bulb reading after completion of turning period: 90-94°F
5. Length of turning period: 18 Days
6. Eggs turned three times daily during turning period
7. Incubator ventilation required on 10th day
8. Egg positioners used until completion of turning point
9. Eggs candled on 7th and 14th days of incubation

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*Recommended by Leahy Manufacturing Company, Inc., Higginsville, Missouri.

APPENDIX C

PARTITIONED CHI-SQUARE TEST USING TCDD VIABILITY DATA IN TABLE 4

	2,4,5-T		TCDD	Mix	Total
Alive	16	+	18	3	37
Dead	20	+	6	21	47
Total	36	+	24	24	84

$$X^2 = \frac{[(34 \times 21) - (26 \times 3]^2 \times 84^2}{60 \times 24 \times 84 \times 37 \times 47}$$

$$= 13.57, \text{ ldf } p < 0.005 \text{ significant}$$

PARTITIONED CHI-SQUARE TEST USING TCDD VIABILITY DATA IN TABLE 3

	2,4,5-T		TCDD	Mix	Total
Alive	16	+	16	3	35
Dead	20	+	8	21	49
Total	36	+	24	24	84

$$X^2 = \frac{[(32 \times 21) - (28 \times 3)]^2 \times 84^2}{60 \times 24 \times 84 \times 35 \times 49}$$

$$= 11.76, \text{ ldf } p < 0.005 \text{ significant}$$

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